Novel ITGB4 Mutations in a Patient with Junctional Epidermolysis Bullosa-Pyloric Atresia Syndrome and Altered Basement Membrane Zone Immunofluorescence for the $\alpha6\beta4$ Integrin

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Immunofluorescence studies of junctional epidermolysis bullosa with pyloric atresia (JEB-PA) have suggested abnormalities in the expression of the $\alpha6\beta4$ integrin, an integral component of hemidesmosomes. In this study, we examined a family with two affected individuals with JEB-PA for mutations in the ITGA6 and ITGB4 genes which encode the $\alpha6$ and $\beta4$ integrin polypeptides, respectively. Mutation detection strategy based on PCR amplification of genomic DNA, followed by heteroduplex analysis and direct nucleotide sequencing, did not reveal sequence variants in ITGA6. Putative pathogenic mutations, however, were identified in both ITGB4 alleles. Specifically, the proband was a compound heterozygote for a 1-bp maternal deletion, 3434delT, and an 8-bp paternal deletion, 4050del8. Both mutations result in a frameshift and premature termination codon downstream from the deletion. At the protein level, immunofluorescence of the skin of the proband revealed negative staining for the integrin $\alpha6$ and markedly reduced staining for the $\beta4$ subunit. Thus, the results support the notion of close association of the $\alpha6\beta4$ integrin subunits and further attest to the critical role of this integrin in providing physiologic stability to the dermal-epidermal junction. ***Key words: blistering skin diseases/hemidesmosomes/$\alpha6\beta4$ integrin mutations. J Invest Dermatol 108:943–946, 1997***

Epidermolysis bullosa (EB) is a clinically and genetically heterogeneous group of blistering diseases characterized by fragility of the skin and mucous membranes (Fine *et al*, 1991; Christiano and Uitto, 1996). On the basis of the level of tissue separation within the dermal-epidermal junction, EB has been traditionally divided into three broad categories: (i) In the simplex forms of EB, tissue separation takes place within the basal keratinocytes. (ii) In the junctional forms of EB (JEB), tissue separation occurs at the level of lamina lucida within the cutaneous basement membrane zone. (iii) In the dystrophic forms of EB, the blistering occurs below the lamina densa within the upper papillary dermis (Uitto and Christiano, 1992). More recently, we have recognized a group of patients with genetic lesions in the genes encoding the protein components of the hemidesmosomes (Tidman and Eady, 1986; McGrath *et al*, 1995, 1996; Chavanas *et al*, 1996; McLean *et al*, 1996; Pulkkinen *et al*, 1996; Smith *et al*, 1996; Gatalica *et al*, 1997).

Manuscript received December 10, 1996; revised February 10, 1997; accepted for publication February 24, 1997.

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Abbreviations: EB, epidermolysis bullosa; JEB-PA, junctional epidermolysis bullosa-pyloric atresia syndrome; CSGE, conformation sensitive gel electrophoresis.

The hemidesmosomes consist of several protein components, including the 230-kDa and 180-kDa bullous pemphigoid antigens, the $\alpha6\beta4$ integrin, and the cytoskeleton-associated protein plectin/HD1 (Green and Jones, 1996). Mutations in the corresponding genes can potentially underlie different hemidesmosomal variants of EB (Uitto *et al*, 1995, 1996). Within each of these categories, several subgroups have been delineated based on the mode of inheritance, extent and severity of the blistering tendency, and association of extracutaneous manifestations (Fine *et al*, 1991).

In a distinct subgroup of EB associated with congenital pyloric atresia, also known as junctional epidermolysis bullosa-pyloric atresia syndrome (JEB-PA) (Nazzaro *et al*, 1990; Lacour *et al*, 1992; Lestringant *et al*, 1992), immunofluorescence studies have suggested abnormalities in the $\alpha6\beta4$ integrin, whereas the expression of other hemidesmosomal proteins appears normal (Gil *et al*, 1994; Phillips *et al*, 1994). Thus, the two genes ITGA6 and ITGB4 encoding the subunit polypeptides of the $\alpha6\beta4$ integrin are the candidate genes for the mutations in JEB-PA. In fact, distinct mutations in a compound heterozygote patient with JEB-PA have been reported in ITGB4, the gene encoding the $\beta4$ integrin polypeptide (Vidal *et al*, 1995).

In this study, we have performed mutation screening on both genes, ITGA6 and ITGB4, in a patient with JEB-PA. This patient was previously shown to have negative immunofluorescence for the $\alpha6$ integrin, and the $\beta4$ integrin immunofluorescence was markedly attenuated (Shimizu *et al*, 1994, 1996).

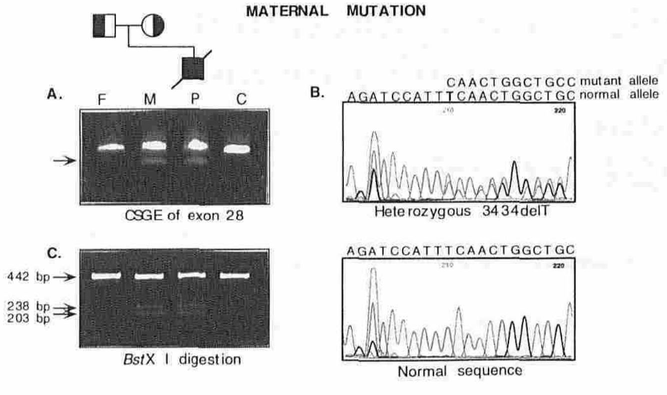

Figure 1. Identification and verification of the maternal mutation in exon 28 of ITGB4. *(A)* Heteroduplex analysis by CSGE revealed a heteroduplex band (arrow) when the PCR product spanning exon 28 of the mother's (M) and the proband's (P) DNA was examined. In contrast, father's (F) DNA revealed the presence of a homoduplex band only, similar to that observed with control DNA (C). *(B)* Direct sequencing of the proband's DNA revealed a heterozygous 1-bp deletion *(upper)*, 3434delT, compared to normal sequence *(lower)*. This deletion resulted in frameshift and premature termination codon 42 bp downstream from the deletion. *(C)* The presence of the mutation was verified by restriction enzyme digestion. The mutation created a new recognition site for *Bst*XI restriction enzyme. Digestion of the mother's and proband's DNA fragmented the mutant allele to 238- and 203-bp bands, whereas the normal allele resisted digestion, *indicating heterozygosity for the mutation. The restriction enzyme digestion* also confirmed that the father was not a carrier of this mutation.

MATERIALS AND METHODS

Clinical The proband with JEB-PA was a neonate born to unrelated clinically unaffected parents. Subsequent pregnancies resulted in a similarly affected female and two younger healthy males. The clinical features and the results of diagnostic immunofluorescence and electron microscopy have been previously described (Shimizu *et al*, 1994, 1996).

DNA for mutation analysis was isolated from the peripheral blood of the parents and unrelated healthy controls by standard techniques (Sambrook *et al*, 1989). The proband's DNA was isolated from a liver specimen that had been obtained at autopsy and preserved in paraffin block.

Detection and Verification of the Mutations For the search of mutations in the ITGA6 and ITGB4 genes (GenBank accession nos. X53586, X52186, and U66529–U66541; Pulkkinen *et al*, unpublished results), individual exons where amplified by polymerase chain reaction (PCR) using total DNA (200 ng) as template. Specifically, for amplification of the exon 28 (nucleotides 3317–3474) in ITGB4, the following primers, placed on flanking intronic sequences, were utilized: sense, 5′-GGCAGGTCT-GAGTTGAATGC-3′; anti-sense, 5′-CTTCCCTCTTTCCCAGCACA-3′.

For amplification of the exon 32 (nucleotides 3977–4108) sequences in ITGB4, the following primers were used: sense, 5′-AGGCCCATGTC-CAGTGAGTG-3′; anti-sense, 5′-TGTCCTCTAGTTGGTGGCCC-3′.

The conditions for PCR were 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 60°C, and 45 s at 72°C.

The PCR products were subjected to heteroduplex analysis using conformation-sensitive gel electrophoresis (CSGE) (Ganguly *et al*, 1993). In case of a heteroduplex, the corresponding PCR product was subjected to direct automated nucleotide sequencing (Applied Biosystems). The mutations identified in the ITGB4 gene were verified at the DNA level by restriction endonuclease digestions. Specifically, the mutation in exon 28, 3434delT (see *Results and Discussion*), created a new restriction enzyme site for the endonuclease *Bst*XI. Thus, the PCR products (control, 442 bp; mutant allele, 441 bp) were digested with this restriction enzyme, and the digestion products were examined on 3% agarose gel. The mutation in exon 32, 4050del8 (see *Results and Discussion*), abolished a site for *Mae*II restriction enzyme. The PCR products representing the normal and mutated alleles, 336 and 328 bp, respectively, were subjected to restriction enzyme digestion and examined on 3% agarose gels.

RESULTS AND DISCUSSION

Clinical and Immunofluorescence Findings The proband was a neonate with extensive blistering at birth associated with pyloric atresia. The patient died on postnatal day 11 from complications of the disease. Subsequently, a similarly affected girl and two phenotypically healthy normal boys were born to the same clinically unaffected parents who were not known to be related. Immunofluorescence studies and electron microscopy of the skin, as detailed elsewhere, were consistent with a junctional form of EB (Shimizu *et al*, 1994, 1996). Briefly, staining with monoclonal antibodies (GoH3 and 450–30A) recognizing $\alpha6$ integrin was entirely negative, and staining for $\beta4$ integrin with three different monoclonal antibodies (439–9B, 450–11A, and 3E1) was markedly reduced. Staining with antibodies recognizing laminin 5, type VII collagen, type IV collagen, and the 230-kDa and 180-kDa bullous pemphigoid antigen epitopes was normal (Shimizu *et al*, 1996). These results suggested that the genes encoding the $\alpha6\beta4$ integrin subunits may contain the mutations resulting in JEB-PA phenotype in this family.

Mutations in the ITGB4 Gene Recent cloning of the ITGA6 and ITGB4 genes (Hogervorst *et al*, 1990, 1991; Tamura *et al*, 1990) encoding the $\alpha6$ and $\beta4$ integrin subunits, respectively, and elucidation of their intron-exon organizations (Pulkkinen *et al*, 1997; Pulkkinen *et al*, unpublished results) has allowed us to develop a mutation detection strategy based on amplification of genomic DNA, followed by heteroduplex analysis using CSGE and direct automated sequencing of the PCR products (see Pulkkinen *et al*, 1996). With this strategy, scanning of the ITGA6 gene did not reveal the presence of any sequence variants in this family (results not shown). Two potentially pathogenic mutations, however, were discovered, one in each allele of the ITGB4 gene of the proband. First, a heteroduplex band with abnormally fast mobility was noted on CSGE when a PCR product spanning exon 28 from the proband's and mother's DNA was analyzed **(Fig 1*A*)**. With the father's DNA, a homoduplex band only, similar to that observed in an unrelated control, was noted. Direct sequencing of the proband's PCR product demonstrating the heteroduplex band revealed a single nucleotide deletion (thymidine) in the position 3434, compared to the control sequence **(Fig 1*B*)**. This mutation,

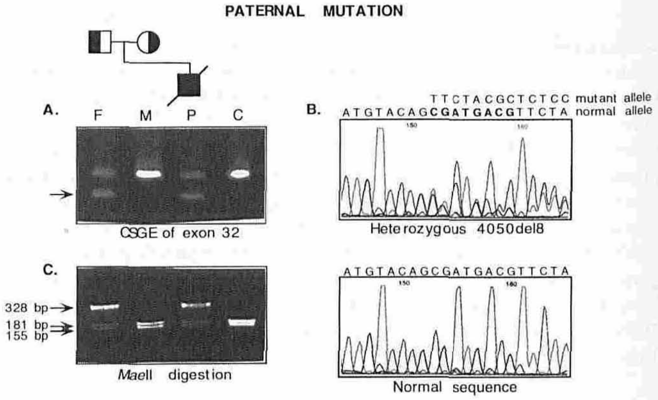

Figure 2. Identification and verification of the paternal mutation within exon 32 of ITGB4. *(A)* Heteroduplex analysis by CSGE revealed the presence of a heteroduplex band (arrow) in father's (F) and the proband's (P) DNA. The mother's (M) DNA revealed the presence of a homoduplex band only, similar to the control DNA (C). *(B)* Direct automated sequencing of the proband's DNA revealed a heterozygous 8-bp deletion, 4050del8, that resulted in frameshift and premature termination codon 44 bp downstream from the deletion. *(C)* The mutation abolished a restriction endonuclease site for *Mae*II restriction enzyme. PCR-amplified DNA (that in the normal and mutated allele gave 336- and 328-bp *restriction endonuclease fragments, respectively)* was digested with restriction enzymes. The 336-bp fragment from the normal allele was digested to 181- and 155-bp fragments, but the 328-bp fragment from the mutated allele resisted digestion. These results confirmed that the father and the proband were heterozygote for this mutation, and the mother contained the normal allele only.

3434delT, created a new restriction enzyme site for the endonuclease *Bst*XI. Thus, digestion of a 441-bp PCR product containing the site of the mutation resulted in the digestion of one allele to 238-bp and 203-bp fragments with the mother's and the proband's DNA, indicating heterozygosity for the presence of this mutation (**Fig 1*C***). As expected, the father's PCR product, 442 bp in size, resisted digestion, similar to that of control DNA. Thus, the patient was heterozygous for a maternally inherited mutation 3434delT within exon 28 of the ITGB4 gene. This deletion resulted in frameshift of translation and a premature termination codon 42 bp downstream from the site of the genetic lesion.

During the search for a putative mutation in the other allele, a heteroduplex band, again with a faster mobility, was noted when the father's and the proband's DNA spanning the exon 32 of the ITGB4 gene was examined on CSGE (**Fig 2*A***). The mother's PCR amplification product showed a homoduplex band only, similar to that observed in control DNA. Direct sequencing of the PCR product demonstrating the heteroduplex band revealed an 8-bp deletion compared to normal sequence (**Fig 2*B***). This mutation, 4050del8, abolished a naturally occurring restriction enzyme site for *Mae*II. Consequently, digestion of PCR products encompassing this region with this endonuclease revealed that the maternal PCR product, 336 bp in size, was digested to 181-bp and 155-bp fragments, similar to that in control DNA (**Fig 2*C***). With the father's and the patient's PCR products, which consisted of two bands, 336 and 328 bp in size, one allele resisted digestion, yielding three bands, 328, 181, and 155 bp in size, and indicating that these individuals where heterozygous for this mutation. This mutation, which resides within exon 32, resulted in frameshift and a premature termination codon 44 bp downstream from the site of the deletion. Thus, the patient was a compound heterozygote for deletion mutations, 3434delT/4050del8, and the parents were heterozygous carriers of the respective mutations in the ITGB4 gene. Finally, screening of 50 unrelated healthy controls did not reveal the presence of either one of these mutations, indicating that they were not common polymorphisms.

Consequences of the Mutations at the Protein Level In this study we have delineated pathogenetic mutations in a patient with JEB-PA. The proband was a compound heterozygote for mutations in the ITGB4 gene and both mutations resulted in frameshift and premature termination codons predicting truncation of the polypeptide within the intracellular domain of the $\beta 4$ integrin. If the truncated polypeptides contain the epitope recognized by the anti-$\beta 4$ integrin antibody, this would explain positive, yet attenuated, immunofluorescence. At the same time, the immunofluorescence for the $\alpha 6$ integrin was entirely negative, yet no mutation could be disclosed in the corresponding gene, ITGA6. Consequently, the absence of the $\alpha 6$ integrin epitope appears to reflect consequences of the primary defect in the ITGB4 gene encoding the $\beta 4$ integrin polypeptide, either because of failure to assemble into hemidesmosomes or to down-regulate this subunit. These data and previous protein studies in this family (Shimizu *et al*, 1996) support the notion that the $\alpha 6$ integrin subunit exists in close association with the $\beta 4$ polypeptide and that the $\beta 4$ integrin subunit is critical for the assembly of hemidesmosomes (Stepp *et al*, 1990). Finally, our results further attest to the critical role of the $\alpha 6 \beta 4$ integrin in providing integrity to the epidermal cell-basement membrane adhesion (Jones *et al*, 1991; Sonnenberg *et al*, 1991; Dowling *et al*, 1996; Georges-Labouesse *et al*, 1996; Van der Neut *et al*, 1996).

We thank the family for their interest in this study. Carol Kelly provided excellent secretarial assistance, and Dr. Hansjürg Alder performed automated nucleotide sequencing. This study was supported in part by the U.S. Public Health Service, National Institutes of Health Grant PO1-AR38923.

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